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RAPID COMMUNICATION

Identification of Determinants of Interaction between CXCR4 and gp120 of a Dual-tropic HIV-1_{DH12} Isolate

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Using a panel of chimeric viruses and their chimeric envelope glycoproteins, we have previously reported that the V1/V2 or the V3 regions of a dual-tropic primary human immunodeficiency virus type 1 (HIV-1) isolate (HIV-1_{DH12}) could individually confer CXCR4 usage when introduced into the backbone of a macrophage-tropic (M-tropic) virus isolate (HIV-1_{AD8}). In this study, chimeric CXCR4–CXCR2 chemokine receptors were employed to identify the determinants involved in the interaction between CXCR4 and the dual-tropic HIV-1_{DH12} gp120. Our results indicate that (i) HIV-1_{DH12} gp120 interacts primarily with the extracellular domains 1 (E1) and 2 (E2) of CXCR4, (ii) the V1/V2 and the V3 regions interact with different domains of CXCR4, and (iii) the V1/V2 region plays a more critical role in the interaction between CXCR4 and HIV-1_{DH12} gp120. Combining our data and those of others suggests that the pattern of CXCR4 usage is highly dependent on HIV-1 isolates. In addition, an M-tropic virus may evolve to become dual-tropic by first acquiring the ability to interact with CXCR4 through the V1/V2 region of gp120.

Human immunodeficiency virus type 1 (HIV-1) enters cells through a series of interactions between its envelope glycoprotein and cellular receptors. The binding of the viral envelope to its primary receptor, CD4, is thought to induce a conformational change in the surface glycoprotein, gp120 (*42*), which allows subsequent interaction with a variety of chemokine receptors (coreceptors; for reviews, see *17, 21, 30, 32, 48*). The cellular tropism of HIV-1 is determined primarily by which chemokine receptor a particular virus strain utilizes (*14, 19, 23, 24, 26, 47*). Macrophage-tropic (M-tropic) strains that are able to infect macrophage, but not T-cell lines, utilize the β-chemokine receptor CCR5. T-cell line tropic (T-tropic) strains utilize the α-chemokine receptor CXCR4 and infect T-cell lines, but not macrophages. Dual-tropic strains can utilize both CCR5 and CXCR4 and are able to infect both cell types. Regardless of cellular tropism, all viruses can infect primary T cells. Although a number of other chemokine receptors have been identified (e.g., CCR2b, CCR3, GPR1, GPR15, STRL33, CCR8, APJ, and CX3CR1), which can function as a coreceptor for different strains of HIV-1 (*13, 14, 16, 20, 23, 28, 40*), their biological significance *in vivo* is not yet clear.

Understanding how viral envelopes and their cellular receptors interact could contribute to the design of effective antiviral agents. Identifying the interacting determinants between gp120 and coreceptors would facilitate this process. Several previous studies have revealed the importance of the gp120 V3 region in determining cellular tropism and coreceptor usage (*8, 9, 14, 15, 18, 29, 33, 35, 43, 45, 47, 49*). More recently we and others have demonstrated that the V1/V2 region, in addition to the V3 region, plays a critical role in coreceptor usage (*10, 39, 44*).

The availability of molecular clones of several chemokine receptors from human and other species has facilitated the identification of receptor domains that interact with HIV-1 gp120. For example, studies with chimeric CCR5 constructs containing CCR2b (*1, 2, 41*) or mouse CCR5 (*4, 34, 38*) suggest that the interaction with gp120 is complex, involving multiple extracellular domains in a highly isolate-dependent manner. Furthermore, fusion mediated by dual-tropic isolates is more sensitive to alterations in CCR5 than is fusion mediated by M-tropic isolates (*22, 34, 41*). Although the interaction between gp120 and CXCR4 is also isolate-dependent, the CXCR4 interacting determinants appear to vary less than CCR5 (i.e., many HIV-1 (*31*) and HIV-2 (*5, 37*) strains preferentially interact with the E3 domain of CXCR4). The E1 domain of CXCR4 is also utilized by some strains of HIV-2 (*5, 36*) and HIV-1 (*5, 31*).

Using chimeric envelope glycoproteins, we have recently reported that either the V1/V2 or the V3 region of

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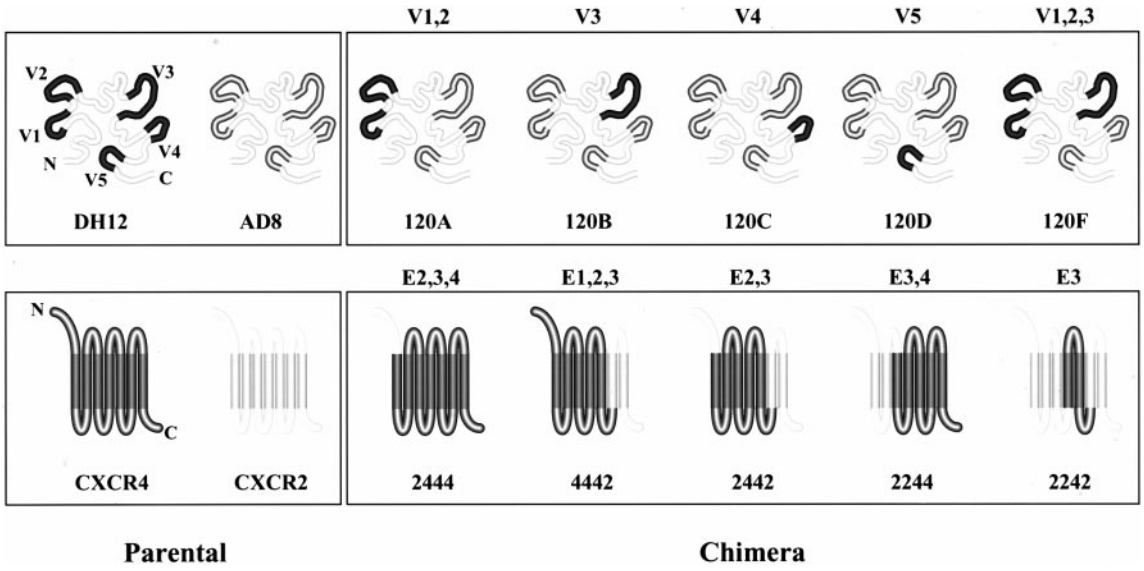

FIG. 1. Schematic diagram of chimeric envelopes and coreceptors. (Top) The parental (DH12 and AD8) and chimeric (120A-D, F) envelopes used in this study. (Bottom) The parental (CXCR4 and CXCR2) and chimeric chemokine receptors. The variable regions and extracellular domains transferred from DH12 to AD8 and from CXCR4 to CXCR2, respectively, are indicated at the top of each panel. For the exact nucleotide position of the junctions for the chimeric proteins, see Cho *et al.* (*11*) and Lu *et al.* (*31*).

a dual-tropic HIV-1 isolate (HIV-1_{DH12}) individually conferred the ability of an M-tropic HIV-1 isolate (HIV-1_{AD8}) to use CXCR4 (*10*). These results raised the possibility that these two variable gp120 regions interacted with different extracellular domains of CXCR4. Using a set of chimeric envelope clones and CXCR4–CXCR2 chimeric chemokine receptors (*31*) we examined the determinants of interaction between CXCR4 and a dual-tropic HIV-1_{DH12} gp120 (Fig. 1).

We selected five chimeric chemokine receptors (i.e., 2444, 4442, 2442, 2244, and 2242) for this study based on their ability to be expressed on the cell surface and to function as coreceptors for HIV-1 gp120 (Fig. 1; (*31*)). A highly sensitive cell–cell fusion assay, utilizing a secreted alkaline phosphatase (SEAP)-chemiluminescence detection system, was used to monitor the envelope–coreceptor interaction. For this purpose, a plasmid encoding a SEAP gene under the control of T7 promoter (pTM-SEAP) was constructed by inserting a *Hin*dIII–*Cla*I fragment from pSEAP-2-Basic (Clontech) into the *Nde*I–*Bam*HI site of pTM–*Nde*I (*12*) subsequent to creating blunt ends with Klenow fragment. pTM-SEAP and the plasmids encoding chimeric envelope glycoproteins (pNVV-B, -C, -D, and -F) were used to construct recombinant vaccinia viruses (vTM-SEAP, vv120Benv, vv120Cenv, vv120Denv, and vv120Fenv, respectively) by methods described previously (*12*). To generate target cells, plasmids encoding wild-type CCR5 (*19*), CXCR4, CXCR2, and CXCR4–CXCR2 chimeric coreceptors (*31*) were transfected into *Mus dunni* mouse fibroblast cells (clone III8C, ATCC) using lipofectin as described by the manufacturer with minor modifications (Gibco BRL). After 4 h of incubation at 37°C, the transfected cells were coinfected with vTF7-3 (*27*) and vCB-3 (*7*) encoding T7 polymerase and human CD4, respectively. To prepare effector cells expressing envelope, *M. dunni* cells were coinfected with vTM-SEAP and one of the following vaccinia viruses expressing HIV-1 envelope: vvADenv (AD8), vvDHenv (DH12), vv120Aenv (*10*); vv120B-D, -Fenv (this study); vBD3 (89.6, (*23*)); vPE-16 (BH8 clone of IIIB, (*25*)); vCB36, vCB28, and vCB43 (RF, JRFL, and Bal, respectively (*6*)). The cells were incubated at 37°C for 5 h, trypsinized, and washed, and duplicate samples containing 5×10^4 each of target and effector cells were mixed in a 96-well plate in the presence of 80 μg/ml of cytosine arabinoside (Ara-C). The cells were incubated for 8 to 10 h at 37°C and the cell–cell fusion activity was monitored by measuring SEAP activity in the culture supernatant using the Great EscAPe SEAP chemiluminescence detection kit (Clontech).

The usage of chimeric coreceptors by the envelopes of the two parental HIV-1 isolates (AD8 and DH12) was first compared to that of other HIV-1 strains (Fig. 2). As expected, the dual-tropic 89.6 gp160 efficiently used both CXCR4 and CCR5 while the T-tropic IIIB gp160 used CXCR4 only. As previously reported (*31*), these two envelopes were able to induce fusion with the 2242 chimera, albeit at low levels, indicating that the E3 domain is involved in CXCR4–gp120 interaction (Fig. 2). In contrast to the results reported by Lu *et al.* (*31*), we observed augmented fusion activity with chimeras 2442 and 2444 compared to 2242 for both 89.6 and IIIB envelopes, suggesting an important contribution by the E2 domain and perhaps a synergy between the E2 and the E3 domains. The inclusion of the E1 domain to 2442 (i.e., 4442) significantly reduced the fusion activity for both of

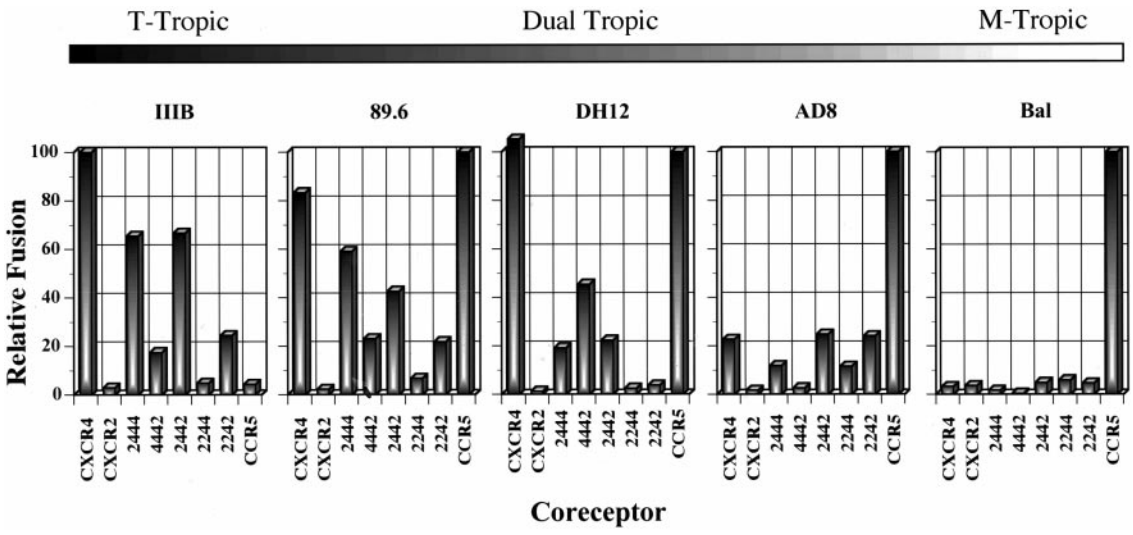

FIG. 2. Comparison of cell–cell fusion activity of HIV-1 envelopes. Cell–cell fusion activity of HIV-1 strains IIIB, 89.6, DH12, AD8, and Bal with chemokine receptors CCR5, CXCR4, and CXCR2 and the CXCR4–CXCR2 chimera was monitored by measuring SEAP activity in the culture supernatant using the chemiluminescence detection method. Chemiluminescence was detected using a 1450 microbeta liquid scintillation counter (Wallac). JRFL and RF exhibited patterns identical to those of Bal and DH12, respectively (data not shown). The fusion activity for CCR5 was designated 100% for all envelopes except for IIIB, for which CXCR4 was used.

these envelope glycoproteins, possibly due to structural incompatibility between the E1 domain of CXCR4 and the E4 domain of CXCR2. For example, the E1 region could be interfering with the interaction between the E2/E3 domain and gp120 in the presence of the E4 domain of CXCR2.

HIV-1_{AD8} is a full-length infectious molecular clone of an M-tropic HIV-1_{ADA} (*46*). The virus is able to replicate in peripheral blood mononuclear cells and monocyte-derived macrophages, but not in T-cell lines (*10*). We and others (*3, 4, 10, 14, 19, 23, 24, 34, 41*) have previously reported that HIV-1_{AD8} uses CCR5 but not CXCR4 for virus entry. Unexpectedly, we detected, using our highly sensitive assay, a low but significant (around 20% of CCR5) level of fusion activity with CXCR4 for AD8 (Fig. 2). This is not an experimental artifact of our assay system since we were unable to detect any fusion with either Bal (Fig. 2) or JRFL (data not shown) gp160s, both of which are M-tropic viruses. A similar level of fusion activity was detected for the chimeric coreceptors 2242, strongly suggesting that the AD8 gp120 interacts with the E3 domain of CXCR4. Interestingly, AD8 exhibited a pattern similar to that of IIIB and 89.6 in that the fusion activity with 4442 was lower than that with 2444 or 2442.

In contrast to IIIB, 89.6, and AD8, DH12 envelope exhibited little or no fusion activity with 2242. Furthermore, the fusion activity was higher for the chimeric coreceptor 4442 than that of either 2444 or 2442. A similar pattern of chimeric coreceptor usage was observed for HIV-1 isolate RF (data not shown). These data suggest that DH12 and RF gp120s interact differently with CXCR4 compared to IIIB, 89.6, and AD8 gp120s and that the E1 and E2 domains of CXCR4 are the primary determinants for interaction with DH12 and RF. This view is consistent with the observation that the RF strain was particularly sensitive to changes in the E1 domain (*31*). Although HIV-1_{RF} has long been considered to be a T-tropic virus, we also found that RF could utilize both CXCR4 and CCR5 (data not shown) as previously reported (*31*).

We have previously demonstrated that either the V1/V2 or the V3 region, but not the V4 or V5 region, of HIV-1_{DH12} gp120 allows HIV-1_{AD8} to use CXCR4 (*10*). Although HIV-1_{AD8} exhibited a low but detectable fusion activity with CXCR4 using a more sensitive assay employed in this study, the completely different pattern of chimeric coreceptor usage compared to HIV-1_{DH12} allowed us to examine which variable regions of HIV-1_{DH12} gp120 interacted with which extracellular domains of CXCR4. As shown in Fig. 3, the chimeric envelopes 120C (V4) and 120D (V5) exhibited a coreceptor usage pattern almost identical to that of the parental AD8 (e.g., low level of CXCR4 usage, ability to utilize 2242, but not 4442). In contrast, the chimeric envelopes 120A (V1/V2), 120B (V3), and 120F (V1-3) were able to efficiently use parental CXCR4 as we have previously shown (*10*). Interestingly, while the chimeric envelopes 120A (V1/V2) and 120F (V1-3) exhibited a pattern identical to that of the parental DH12 gp120, 120B (V3) exhibited a hybrid phenotype: (i) fusion activity with 2242 (AD8 phenotype); (ii) good fusion activity with 4442 (DH12 phenotype); and (iii) higher fusion activity with 2444 and 2442 compared to 4442 (AD8 phenotype). In fact, the general pattern looked very similar to that of 89.6 (Fig. 2). These results demonstrate that the V1/V2 region of DH12 gp120 interacts with the E1 and E2 domains of CXCR4, the V1/V2 and the V3 regions

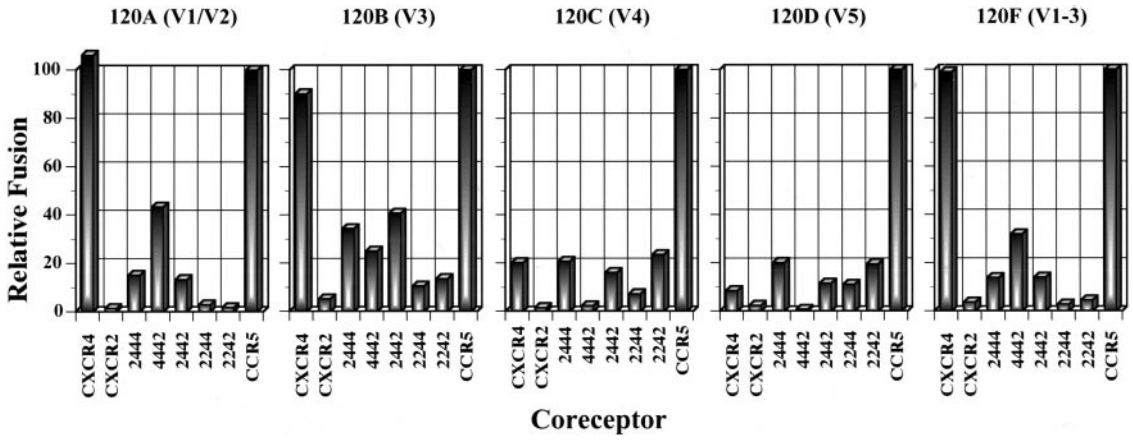

FIG. 3. Relative cell–cell fusion activity of chimeric envelopes. Chimeric envelopes 120A (V1/V2), 120B (V3), 120C (V4), 120D (V5), and 120F (V1-3) were tested for fusogenic activity against the chimeric CXCR4–CXCR2 coreceptors. The fusion activity for CCR5 was designated 100% for all envelopes.

interact with CXCR4 differently, and the V1/V2 region plays a more critical role than the V3 region in the interaction between CXCR4 and HIV-1_{DH12} gp120. The chimeric envelope 120B (V3), but not 120A (V1/V2), retained the ability to utilize 2242, suggesting, although it does not prove, that the V1/V2 region of AD8 gp120 may interact with the E3 domain of CXCR4.

Although the use of chimeric proteins has led to an understanding of the structural and functional properties of many proteins, there are undoubtedly limitations that arise simply because some combinations are structurally incompatible and render the chimera nonfunctional. Although many more chimeric coreceptors were generated between CXCR4 and CXCR2 (*31*), we used only five that could be expressed well on the cell surface. Because some chimeric proteins may be only partially functional, greater emphasis should be placed on the positive fusion activity rather than the negative results. In addition, what should be emphasized is how the overall pattern of chimeric coreceptor usage of one isolate compares with that of other viruses. For example, while HIV-1 strains IIIB and 89.6 could utilize 2242, DH12 and RF could not (Fig. 2; and data not shown). Thus, it can be concluded that the E3 domain plays a more important function for IIIB and 89.6 than for DH12 or RF. While the fusion activity with 4442 was significantly lower than 2444 or 2442 for IIIB and 89.6, the inverse was true for DH12 and RF, indicating that the E1 domain plays a critical role for the latter two strains (Fig. 2). Combining the results from this and previously reported studies thus far, three general patterns emerge with respect to how different isolates of HIV-1 and HIV-2 envelopes interact with CXCR4: utilization of (i) the E2 and E3 domains (e.g., HIV-1 IIIB and 89.6; (*31*) and this study); (ii) the E1 and E2 domains (e.g., HIV-1 DH12 and RF; (*31*) and this study); and (iii) the E1 and E3 domains (e.g., HIV-1 NDK and HIV-2 ROD; (*5, 36, 37*)).

We have employed a highly sensitive cell–cell fusion assay in this study. High sensitivity was achieved by using chemiluminescence detection of SEAP and using recombinant vaccinia viruses to express all of the components involved in fusion (except for the coreceptors), which is by far much more efficient than DNA transfection. The level of expression of the various components in a cell–cell fusion assay is likely much greater than the level observed *in vivo.* Thus, although cell–cell fusion assay has been shown to correlate well with virus infectivity assays in many previously reported studies and the results are biochemically valid, they should be interpreted with some caution. It is likely that the detection limit of the fusion assay is much lower than that of the virus infectivity assay in which case viral envelope–cellular receptor interaction could be detected by the former but not the latter assay. Thus, it is not surprising to detect a low fusion activity of AD8 envelope with CXCR4 (Fig. 2) although HIV-1_{AD8} could not infect the MT4 T-cell line and its infection of PBMC could be completely inhibited by RANTES and MIP-1β (*10*). Our observation raises the question of how to properly classify cellular tropism of HIV isolates. Cellular tropism should be determined by whether or not a virus isolate can achieve an efficient spreading infection in biologically relevant cells. Coreceptor usage analyses using a cell–cell fusion assay are performed to understand the complex biochemical interactions between viral envelope and cellular receptors. Thus, it may be more appropriate to classify HIV-1_{AD8} as an M-tropic (R5) virus despite a low level of fusion activity with CXCR4.

The results in this study show that transfer of either the V1/V2 or the V3 regions of DH12 gp120 in the background of AD8 envelope led to efficient utilization of CXCR4 by AD8 (Fig. 3). The results further suggest that the interaction between V1/V2 and CXCR4 is more important than that between V3 and CXCR4 for the HIV-1_{DH12} envelope. Although the V3 region of gp120 has recently been shown to play a critical role for strictly T-tropic HIV-1

isolates in determining cellular tropism and CXCR4 usage (*45*), it is possible that the V1/V2 region may play a more important function for dual-tropic isolates. In this regard, it is interesting to note that Smyth *et al.* (*44*) have reported that the regions outside of the V3-5 (presumably the V1/V2 region) determine CXCR4 utilization for HIV-$1_{89.6}$. Furthermore, a B-cell epitope of a highly potent neutralizing antibody elicited in a chimpanzee infected with HIV-1_{DH12} consists of parts of V1/V2, but not the V3 region (manuscript in preparation).

Dual-tropic viruses may represent evolutionary intermediates between M-tropic and T-tropic isolates during the course of AIDS pathogenesis *in vivo*. It has been proposed that dual-tropic viruses evolve from M-tropic isolates by acquiring the ability to use CXCR4 while retaining the ability to interact with CCR5 (*31*). It is possible that the HIV-1_{AD8} isolate may represent an early variant of an M-tropic virus that has just acquired the ability to use CXCR4. The fusion activity with 2242 was comparable with that of CXCR4, suggesting that AD8 utilizes primarily the E3 domain (Fig. 2). Replacement of the V1/V2 region of AD8 gp120 with that of DH12 (120A), but not the V3 region (120B), eliminated the ability of the chimeric envelope to utilize 2242, suggesting that the V1/V2 region of AD8 gp120 might interact with the E3 domain of CXCR4. Since the V1/V2 region seems to play a crucial role in utilizing CXCR4 for the dual-tropic HIV-1 isolates examined thus far (i.e., DH12 and 89.6 (*44*)), one could speculate that M-tropic viruses initially become dual-tropic by acquiring the ability to interact with CXCR4 through its V1/V2 region while the V3 region maintains its ability to utilize CCR5. The V1/V2 region may interact with either the E1 (e.g., DH12 and RF) or the E3 (e.g., 89.6) domains of CXCR4. Subsequently, the V3 region of gp120 could accumulate mutations that could strengthen the interaction between viral envelope and CXCR4. Alternatively, the V1/V2 region, the V3 region, or both regions could coevolve in a nonsequential manner that gradually alters the coreceptor usage pattern of an M-tropic virus to that of a T-tropic virus. Clearly, there have not been enough studies on identification of determinants of interaction for dual-tropic viruses to definitively state one way or the other. Future studies using chimeric envelopes generated between AD8 and strictly CCR5 utilizing envelopes (e.g., Bal or JRFL) may provide better understanding of viral evolution with respect to cellular tropism and coreceptor usage.

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